D5

ELSEVIER

Biochimica et Biophysica Acta 1417 (1999) 37–50

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

A transfection compound series based on a versatile Tris linkage

Fiona H. Cameron *, Minoo J. Moghaddam, Vera J. Bender, Robert G. Whittaker, Margaret Mott, Trevor J. Lockett

CSIRO Division of Molecular Science, Sydney Laboratory, 2 Richardson Pl., 103 Delhi Rd, North Ryde, 2113, Sydney, Australia

Received 28 September 1998; received in revised form 30 November 1998; accepted 1 December 1998

Abstract

The family of cationic lipid transfection reagents described here demonstrates a modular design that offers potential for the ready synthesis of a wide variety of molecular variants. The key feature of these new molecules is the use of Tris as a linker for joining the hydrophobic domain to a cationic head group. The molecular design offers the opportunity to conveniently synthesise compounds differing in charge, the number and nature of hydrophobic groups in the hydrophobic domain and the characteristics of the spacer between the cationic and hydrophobic moieties. We show that prototype reagents of this design can deliver reporter genes into cultured cells with efficiencies rivalling those of established cationic lipid transfection reagents. A feature of these reagents is that they are not dependent on formulation with a neutral lipid for activity.

Keywords: Transfection; Cationic lipid; Mammalian cell; Tris; Chinese hamster ovary; Cytofectin; Lipopeptide

1. Introduction

It is well established that cationic lipids can be used to successfully deliver genes and oligonucleotides into a wide variety of different cell types in vitro. More recently they have been used for the delivery of therapeutic genes in a number of gene therapy clinical trials.

Although less efficient than viruses at delivering a nucleic acid load into cells, cationic lipids are flexible delivery agents facilitating the transfer of anything from oligonucleotides to large gene expression cassettes into cells and tissues without raising an immune response or risking oncogenicity resulting from the inadvertent formation of recombinant virus. Thus despite the drawbacks, the benefits of cationic lipid systems have justified widespread research being carried out on these compounds as viable alternatives to viral delivery (for reviews see [1–8]).

In this paper we describe a new class of cationic lipids which offers a chemical versatility not available in other compounds of this type. These compounds take advantage of the observation that the amino group of the common buffer molecule Tris can, under certain conditions, be induced to form an

Abbreviations: A, alanine; Boc, *tert*-butyloxycarbonyl; Cbz, carbobenzyloxy; Boc(K-Cbz), α-*tert*-butyloxycarbonyl-ε-carbobenzyloxy-lysine; Cbz-A-OMe, carbobenzyloxy-alanine methyl ester; DCM, dichloromethane; DCCD, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DIEA, diisopropylethylamine; DMAP, dimethylaminopyridine; DMF, dimethylformamide; DMSO, dimethylsulphoxide; DSC, disuccinimidylcarbonate; HOSu, *N*-hydroxysuccinimide; Pd/C, palladium/carbon; K, lysine; P, palmitoyl (hexadecanoyl); TBA, *tert*-butanol; TFA, trifluoroacetic acid; T, Tris(hydroxymethyl)aminomethane; THF, tetrahydrofuran

* Corresponding author. Fax: +61 (2) 94905005; E-mail: fiona.cameron@molsci.csiro.au

0005-2736/99/$ – see front matter
PII: S0005-2736(98)00248-X

amide linkage with the α-carboxyl group of an amino acid or peptide [9]. These conjugated cationic molecules have a number of features in common with classical lipidic molecules used for transfection. That is, they are characterised by a polar cationic head group attached to a number of long chain fatty acyl groups.

We demonstrate that by attaching a cationic peptide to the amino group of Tris and by forming ester linkages with the long chain fatty acyl molecule palmitoyl to the hydroxyls of the Tris linker, prototype cationic lipopeptides can be formed that efficiently deliver DNA containing a β-galactosidase reporter gene cassette into cells in culture. Further we demonstrate that varying the number of palmitoyl groups in the hydrophobic domain dramatically alters the transfection activity of these compounds. Transfection activity was not dependent on formulation with a neutral lipid which significantly simplifies their preparation and use as transfection reagents.

2. Materials and methods

2.1. Preparation of cationic lipids

2.1.1. Chemicals and solvents

Boc(K-Cbz) was purchased from Peptide Institute (Osaka, Japan) and DSC was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All the amino acids were in L-configuration and were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. All solvents were of analytical grade and were used as purchased.

2.1.2. Thin layer chromatography (TLC)

TLC was performed on Alufolein Silica gel 60 F_{254} plates (Merck) in the following solvent systems: R_f^1, chloroform/methanol/acetic acid: 95:5:3; R_f^2, chloroform/methanol/triethylamine: 95:7:3. Compounds with UV absorbance were visualised under UV light and then were sprayed with phosphomolybdic acid (4% ethanol solution) followed by heating at 120°C. Compounds containing unprotected amine groups were visualised by ninhydrin spray followed by heating at 120°C.

2.1.3. High performance liquid chromatography (HPLC)

Analytical HPLC was carried out on Millipore Waters HPLC equipment (Waters Chromatography Division of Millipore, Milford, MA, USA), comprising a 6000A series solvent delivery system with an automated gradient controller and Model 746 Data Module. The chromatography was carried out with a Novapak C_{18} reverse phase column (100×8 mm). The peptides and peptide-Tris conjugates were analysed on a linear gradient elution from 24 to 80% acetonitrile with 0.1% TFA within 5 min at a flow rate of 2 ml/min (System A; R_t^A) Detection was carried out at 260 nm using a Waters Lambda Max 480 LC spectrophotometer.

The lipopeptide conjugates were analysed on a C_{18} column with a linear gradient from 40% water, 50% acetonitrile and 10% THF to 50% acetonitrile, 50% THF with 0.1% TFA within 5 min at a flow rate of 2 ml/min (System B; R_t^B).

2.1.4. Preparative HPLC

Separations were carried out on a Millipore Waters DeltaPrep 4000 HPLC using a PrePak C_{18} reverse phase column (100×40 mm) eluted with a linear gradient with the same eluent buffer systems as described above for the analytical HPLC (without TFA) at a flow rate of 20 ml/min.

Preparative silica column flash chromatography was performed on silica 230–400 mesh ASTM (Merck).

2.1.5. Nuclear magnetic resonance (NMR)

^{1}H NMR spectra were recorded with a 200 MHz Brucker spectrophotometer at the probe temperature of 298 K.

2.2. Compound syntheses

2.2.1. α-Boc(ε-Cbz)-lysyl-(ε-Cbz)-lysine [Boc(K-Cbz)$_2$OH]; compound I

Boc(K-Cbz), (2.85 g, 7.5 mmol) was dissolved in 40 ml acetonitrile. HOSu (1 g, 8.7 mmol) was added to the solution and it was cooled to 0°C. DCCD (1.55 g, 7.5 mmol) dissolved in 10 ml acetonitrile was added dropwise into the reaction mixture. The solution was stirred at 0°C for 1 h, transferred to

room temperature, then stirred a further 3 h to produce the hydroxysuccinimide ester in 93% yield by HPLC. DCU precipitate was removed by filtration. To the filtrate was added 20 ml of 50:50 (v/v) water/acetonitrile containing 2.1 g (7.5 mmol) α-amino (K-Cbz) and 1.7 ml DIEA (10 mmol). The reaction mixture was stirred at room temperature overnight. Solvent was removed under reduced pressure and the resulting oily residue was dissolved in ethyl acetate and washed with acid, base and water. The ethyl acetate phase was dried over sodium sulphate and evaporated to dryness. The residue was triturated with diethyl ether to obtain 4.5 g of compound I in 93% yield, R_f^1: 0.52, R_t^A: 7.53 min; ^{1}H NMR: δ (DMSO-d_6, ppm), 1.39 (9H, s, Boc$(CH_3)_3$), 1.4–1.8 (12H, brs, β, γ, δ CH_2), 2.99 (4H, brs, ε-CH_2), 3.95 (1H, m, α-CH), 4.14 (1H, m, α-CH), 5.03 (4H, s, Ar-CH_2), 6.89 (1H, d, α-urethane NH, J = 7.5 Hz), 7.25 (2H, t, ε-urethane NH), 7.41 (10H, m, Ar(H)), 7.95 (1H, d, amide NH, J = 8.5 Hz).

2.2.2. α-Amino(ε-Cbz)-lysyl-(ε-Cbz)-lysine ($TFA^-.\alpha$-NH_3^+ (K-Cbz)$_2$OH); compound II

Compound I (3.8 g, 5.85 mmol) was dissolved in 10 ml DCM, and cooled to 0°C. TFA (10 ml) was added and the reaction mixture stirred at 0°C for 10 min. After stirring for a further 50 min at room temperature the solvent and the excess TFA were removed by evaporation and the oily residue triturated with diethyl ether yielding 3.9 g of compound II; R_f^2: 0.19, R_t^A: 5.78 min.

2.2.3. α-Boc(ε-Cbz)-lysyl-(ε-Cbz)-lysyl-(ε-Cbz)-lysine [Boc(K-Cbz)$_3$OH]; compound III

Boc(K-Cbz) (2.23 g, 5.85 mmol) was activated by HOSu and DCCD as described above. DCU was removed by filtration and the filtrate added to 3.9 g of compound II. DIEA (2 ml, 11.7 mmol) was added to the reaction mixture which was then stirred overnight at room temperature. The solvent was evaporated and the residue dissolved in ethyl acetate and washed with acid, base and water. The ethyl acetate solution was dried over sodium sulphate. By concentrating the ethyl acetate solution white crystals were formed which were collected by filtration and dried to give 3 g of pure compound III as determined by HPLC. The filtrate was evaporated to dryness and the residue purified on the preparative HPLC C18 column to give a further 0.7 g of pure compound III (total yield: 87%), R_f^1: 0.36, R_t^A: 7.92 min; ^{1}H NMR: δ (DMSO-d_6, ppm), 1.39 (9H, s, Boc$(CH_3)_3$), 1.4–1.8 (18H, brs, β, γ, δ CH_2), 2.99 (6H, brs, ε-CH_2), 3.95 (1H, m, α-CH), 4.14 (1H, m, α-CH), 4.34 (1H, m, α-CH), 5.03 (6H, s, Ar-CH_2), 6.8 (1H, d, α-urethane NH, J = 7.5 Hz), 7.25 (3H, t, ε-urethane NH), 7.41 (15H, m, Ar(H)), 7.80 (1H, d, amide NH, J = 8 Hz), 8.15 (1H, d, amide NH, J = 8 Hz).

2.2.4. N-(Cbz-Alanyl) Tris(hydroxymethyl)aminomethane (Cbz-AT); compound IV

Cbz-alanine methyl ester (9 g, 0.038 mole) was dissolved in 100 ml of DMF and Tris (36.5 g, 0.3 mole) in 300 ml of H_2O and 350 ml of DMF were added to the reaction mixture and incubated at 60°C for 72 h. The solvents were removed under reduced pressure and the oily residue redissolved in ethyl acetate and washed with water. The ethyl acetate phase was dried over anhydrous sodium sulphate and evaporated to dryness to obtain 4 g of pure compound IV. Subsequent extraction of the water phase with ethyl acetate yielded a further 2.8 g of the pure compound representing a total yield of 55%. R_t^A: 3.96 min; ^{1}H NMR: δ (DMSO-d_6, ppm), 1.2 (3H, d, alanine(CH_3), J = 6 Hz), 3.63(6H, d, Tris(CH_2), J = 4 Hz), 4.14(1H, m, α-CH), 5.03 (2H, s, Ar-CH_2), 7.15 (1H, s, amide-NH), 7.41 (5H, m, Ar(H)), 7.5 (1H, d, urethane NH, J = 7.5 Hz).

2.2.5. N-(Alanyl) Tris(hydroxymethyl)aminomethane (AT); compound V

Compound IV (4 g, 0.012 mole) was dissolved in 60 ml of mixed solvent of DCM/methanol (50:50), then hydrogenated overnight at 40 psi in a Parr Hydrogenator using 10% Pd/C. Pd/C was removed by filtration and the solvents evaporated under reduced pressure to give 2.2 g of compound V in 94% yield. The removal of the Cbz group was confirmed by ^{1}H NMR spectroscopy (by disappearance of chemical shifts at 5.03 and 7.41 ppm).

2.2.6. N-{α-Boc[(ε-Cbz) Lysyl]$_3$-alanyl}-Tris(hydroxymethyl)aminomethane [Boc(K-Cbz)$_3$-AT]; compound VI

Compound III (1 g, 1.2 mmol) was dissolved in

40 ml DMF and DSC (0.92 g, 3.6 mmol) added. After adding DIEA (0.2 ml, 1.2 mmol) and stirring at room temperature for 1 h the activated ester of compound III formed with a 78% yield as determined by HPLC. Compound V (0.46 g, 2.4 mmol) was added to the reaction mixture and the pH adjusted to 8 by adding 0.6 ml of DIEA. The formation of the title compound was followed by HPLC. After 2 h the activated ester was almost fully utilised being either coupled to compound V to form compound VI or hydrolysed to compound III. The total yield of compound VI was 52% by HPLC. Preparative HPLC yielded 270 mg of pure compound with R_t^A: 7.4 min; ^{1}H NMR: δ (DMSO-d_6, ppm), 1.1–1.4 (12H, b, alanine(CH_3), Boc(CH_3)$_3$), 1.4–1.9 (18H, brs, β, γ, δ CH_2), 2.97 (6H, brs, ε-CH_2), 3.53 (6H, d, Tris(CH_2), J=4 Hz), 3.75–4.43 (4H, brs, α-CH), 3.89 (3H, t, OH), 5.08 (6H, s, Ar-CH_2), 6.8 (1H, d, α-urethane NH, J=7.4 Hz), 7.19 (3H, t, ε-urethane NH), 7.41 (15H, m, Ar(H)), 7.80 (1H, d, amide NH, J=8 Hz), 8.15 (1H, d, amide NH, J=8 Hz), 8.35 (1H,. d, amide NH, J=8 Hz).

2.2.7. *N-{α-Boc [(ε-Cbz) Lysyl]$_3$-alanyl}Tris(O, O′, O″-mono, di- and tri-hexadecanoyl-hydroxymethyl)aminomethane [Boc(K-Cbz)$_3$-AT(P)$_n$, n=1, 2, 3]; compounds VII-a,b,c*

Compound VI (173 mg, 0.160 mmol) was dissolved in 3 ml DCM and 1 ml DMF. Palmitic acid (89.6 mg, 0.35 mmol) and a catalytic amount of DMAP were added to the reaction mixture. DCCD (72 mg, 0.35 mmol) dissolved in 2 ml DCM was added dropwise into the reaction mixture and stirred at room temperature overnight. The reaction produced a mixture of mono-, di- and tri-palmitoyl representing 17%, 40% and 43% of the final product as determined by HPLC (System B). The solvents were evaporated to dryness and the residue redissolved in DCM. DCU was removed by filtration and the filtrate washed with sodium bicarbonate (5%) and water. The products were separated by preparative HPLC followed by silica chromatography (DCM/MeOH) yielding the monopalmitoyl (17 mg, R_t^B: 7.63 min) dipalmitoyl (76 mg, R_t^B: 8.65 min), and tripalmitoyl (63 mg, R_t^B: 9.29 min); ^{1}H NMR of the tripalmitoyl compound: δ (chloroform-d, ppm):0.8–0.95 (9H, t, 3CH_3), 1.3–1.47 (84H, m, Boc(CH_3)$_3$, palmitoyl (CH_2), alanine (CH_3)), 1.47–2.0 (18H, brs, β, γ, δ CH_2), 1.83 (6H, t, β-CH_2), 2.28 (6H, t, α-CH_2), 3.09 (6H, brs, ε-CH_2), 3.97 (2H, m, α-CH), 4.29 (1H, m, α-CH), 4.35 (6H, s, Tris(CH_2)), 4.47 (1H, t, αCH), 5.04 (6H, s, Ar-CH_2), 5.56 (1H, d, amide NH, J=7 Hz), 5.7 (1H, d, amide NH, J=7.5 Hz), 5.78 (1H, d, amide NH, J=7.5 Hz), 6.92 (1H, d, α-urethane NH, J=7.4 Hz), 7.19 (3H, t, ε-urethane NH), 7.35 (15H, m, Ar(H)).

2.2.8. *[(Lysyl)$_3$-alanyl] Tris(O, O′, O″-mono-, di- and tri-hexadecanoyl-hydroxymethyl)aminomethane [K$_3$AT(P)$_n$, n=1, 2, 3); compound VIII-a,b,c*

Compound VII-c with $n=3$ (45 mg) was dissolved in DCM (2 ml) and cooled to 0°C. TFA (2 ml) was added to remove the Boc group at 0°C for 10 min and room temperature for 30 min. The solvent and the excess TFA were removed thoroughly by repeated co-evaporation with diethylether and precipitated from diethylether. Removal of the Boc protecting group was confirmed by ^{1}H NMR spectroscopy. The precipitate was then dissolved in a mixed solution of DCM/methanol (50:50, 4 ml) and hydrogenated for 2 h at 40 psi in a Parr Hydrogenator using 10% palladium/carbon to remove the Cbz groups. The removal of the Cbz groups was confirmed by ^{1}H NMR spectroscopy (by disappearance of chemical shifts at 5.04 and 7.35 ppm).

Compounds VII-a and VII-b were deprotected by the same method as described above. Finally the compounds were freeze dried from TBA and stored at 4°C.

For transfection, freeze dried compounds were dissolved to 20 mM in ethanol and then diluted to 10 mM with sterile water. Working stocks (2 mM), prepared by further dilution of the 10 mM stock in sterile water, were stored at 4°C.

2.3. *Plasmids*

pPGKlacZNLS [10], pCIGAL and pSVGAL [11] are all mammalian expression vectors carrying the gene cassette encoding β-galactosidase production. The promoters used were mouse PGK, CMV immediate early and SV40 early, respectively. In addition, the PGK plasmid contains an SV40 nuclear localisation signal which directs the LacZ protein to the nucleus. pCIGal was engineered on the vector back-

bone pCI (Promega) as follows. The *lacZ* fusion gene introduced into the pCI vector was assembled by ligating the 1.4 kb *Hin*dIII-*Eco*RV 5′ terminal fragment of the *lacZ* gene from pSVGal to the 1.96 kb *Eco*RV-*Bam*HI 3′ terminal fragment of the *lacZ* gene from pMC1817 [12] in a 3-way ligation with *Hin*dIII-*Bam*HI cut Bluescript SK^+ (Clontech) to produce pBstlacZ. The entire *lacZ* gene from pBstlacZ was then released as a 3.3 kb *Xho*I-*Xba*I fragment and ligated to *Xho*I-*Xba*I cut pCI. The resultant plasmid pCIGal carried the *lacZ* gene under the control of the CMV immediate early promoter. As with pSVGal, the reporter gene in the pCIGal expression plasmid encodes a fusion protein containing 41 N-terminal amino acids from the *Escherichia coli* xanthine guanine phosphoribosyl transferase (XGPRT) enzyme, 29 N-terminal amino acids from the *E. coli* tryptophanyl tRNA synthetase enzyme and the C-terminal catalytic fragment from the *E. coli* β-galactosidase enzyme [13].

2.4. Cell culture

Chinese hamster ovary (CHO) cells were cultured in minimal essential medium with Earle's salts (EMEM) with 10% foetal calf serum (FCS) at 37°C and 5% CO_2. Mouse 3T3 cells were grown similarly but in Dulbecco's modified Eagle's medium with 10% FCS.

2.5. Transfection and assay

Cells were seeded in an 8×9 array in 96-well microtitre trays at 1×10^4/well (CHO) or 2×10^4/well (3T3) and allowed to adhere overnight.

In CHO cells, pPGKlacZNLS and Bluescribe (Clontech) were used at a 1:3 ratio. Bluescribe was used as a non-coding DNA to make up the required plasmid levels where expression from transfected cells was otherwise too high to give a linear readout in the β-galactosidase assay. In 3T3 cells, in order to maximise expression of the transgene in this lower expressing cell line, two plasmids pCIGAL and pSVGAL were used, mixed at a 1:1 ratio.

Transfection was carried out essentially as described by Felgner et al. [14]. Briefly, in separate polystyrene dilution plates, plasmid encoding β-galactosidase was doubly diluted down from 2 μg/well (60.6 μM nucleotide in 100 μl final complex volume), and test transfection reagent was separately diluted across eight wells of the plate to give either 168 μM or 84 μM highest concentration (in 100 μl final complex volume) in serum free medium, with column 9 left free of lipid to provide a DNA-alone control. DNA dilutions were transferred across into the transfection reagent dilutions using a multichannel pipette. After 10 min at room temperature, 100 μl of the mix was transferred into the wells of plates of cells washed free of serum immediately prior to transfer. After 3–6 h of incubation (generally 4 h) an equal volume (100 μl) of full medium (10% FCS) was added to each well and allowed to incubate overnight under standard conditions. In experiments comparing different cells types, medium with 20% FCS was added to bring the final concentration of FCS to 10% for overnight incubation. Medium was exchanged for fresh full medium and allowed to further incubate until 48 h post transfection.

Cell viability was determined using the dye MTS (Promega) according to the manufacturer's instructions. The colour was allowed to develop until an $OD_{492/650}\sim1$. After reading the colour change at 492/650 nm, the cells were washed with PBS and frozen at −70°C in lysis buffer (1 M Tris pH 8, 0.1% Triton X-100) for subsequent β-galactosidase assay of the lysate as described [14] using the CPRG substrate (Boehringer Mannheim). An *E. coli* β-galactosidase standard, at 1500 units/ml (No. 105031 Boehringer Mannheim) was used for conversion of absorbance data to β-galactosidase units.

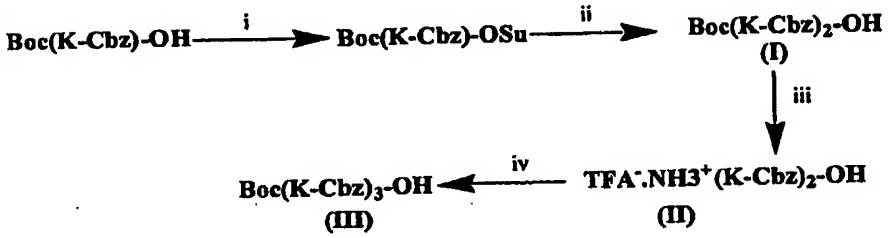
2.6. Analysis of data

The 'peak' level of transfection is defined as the β-galactosidase activity observed in that well of the 8×8 array displaying the highest levels of reporter gene expression. The 'summed' data are calculated by summing the β-galactosidase activity observed in each well of the 8×8 array.

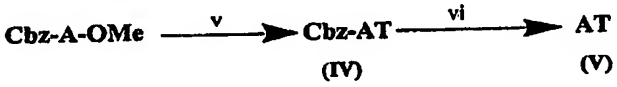
3. Results

3.1. Generation of three test compounds from one synthesis

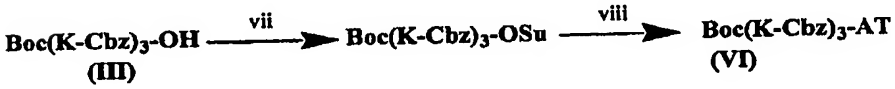
The synthesis of $K_3AT(P)_n$, $n=1$, 2, 3, included



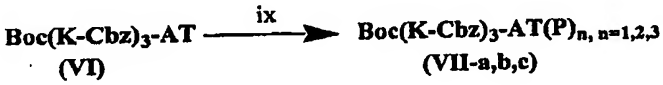
Stage 1: Synthesis of the building block Boc(K-Cbz)$_3$OH: i: DCCD, HOSu, Acetonitrile ii: NH$_2$-(K-Cbz)-OH, iii: TFA/DCM (50/50 V/V), Boc(K-Cbz)-OSu



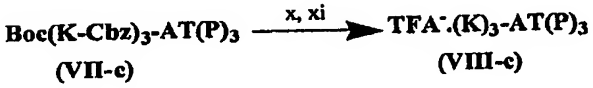
Stage 2: Synthesis of the building block A-T; v: Tris, DMF, H$_2$O, vi: Pd/C, DCM/MeOH



Stage 3: Synthesis of Boc(K-Cbz)$_3$-AT; vii: DSC, DMF, viii: AT



Stage 4: Synthesis of Boc(K-cbz)$_3$-AT(P)n vii: Palmitic acid, DCCD, DMAP, DCM/DMF



Stage 5: Removal of the protecting group ; x: TFA/DCM, xi: pd/C, DCM/MeOH, H2

Fig. 1. Synthesis pathway of $K_3AT(P)_n$.

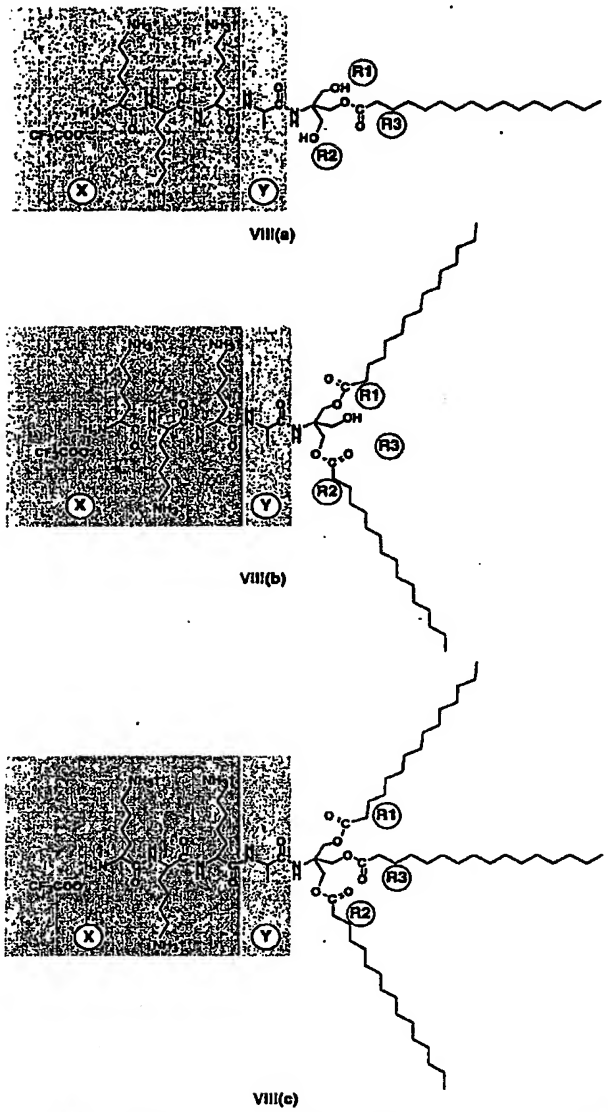


Fig. 2. Structures of $K_3AT(P)_n$. X, charged peptide domain; Y, amino acid spacer domain, linked through Tris to R1–R3 fatty acyl derivative domain. Displayed from top: a, K_3ATP_1; b, K_3ATP_2; c, K_3ATP_3. Roman numerals refer to positions in synthesis pathway (see Fig. 1).

five stages as shown in Fig. 1. The first and second stages involved preparation of Boc(K-Cbz)$_3$ and the coupling of alanine to Tris (AT). The synthesis of Boc(K-Cbz)$_3$ was carried out by classical solution methods [15]. Cbz-Ala-Tris was prepared as described by Whittaker et al. [9] with some modification in the purification process. The third stage involved activating the C-terminal carboxyl group of the Boc(K-Cbz)$_3$, coupling to AT and purification of the product by preparative HPLC. The fourth stage of the preparation included conjugation of the palmitic acid to the Boc(K-Cbz)$_3$ AT. By adding differ-

ent ratios of the palmitic acid and the coupling reagent (DCCD) different populations of the three conjugates BOC(K-Cbz)$_3$ AT(P)$_n$, $n=1$, 2, 3, were generated. The three conjugates were then separated on a preparative HPLC (C18 column) followed by silica-gel chromatography to obtain high purity conjugates. In the last stage each conjugate was deprotected from Boc and Cbz groups. The purity of the intermediates and the final products were all checked by TLC, HPLC, and ^{1}H NMR.

Each of these molecules was characterised by the presence of four positive charges at the hydrophilic end, and palmitoyl ($n=1$–3) at the hydrophobic end. The structures for these molecules are shown in Fig. 2. In this figure the three different domains which can be readily varied are designated 'X' (cationic do-

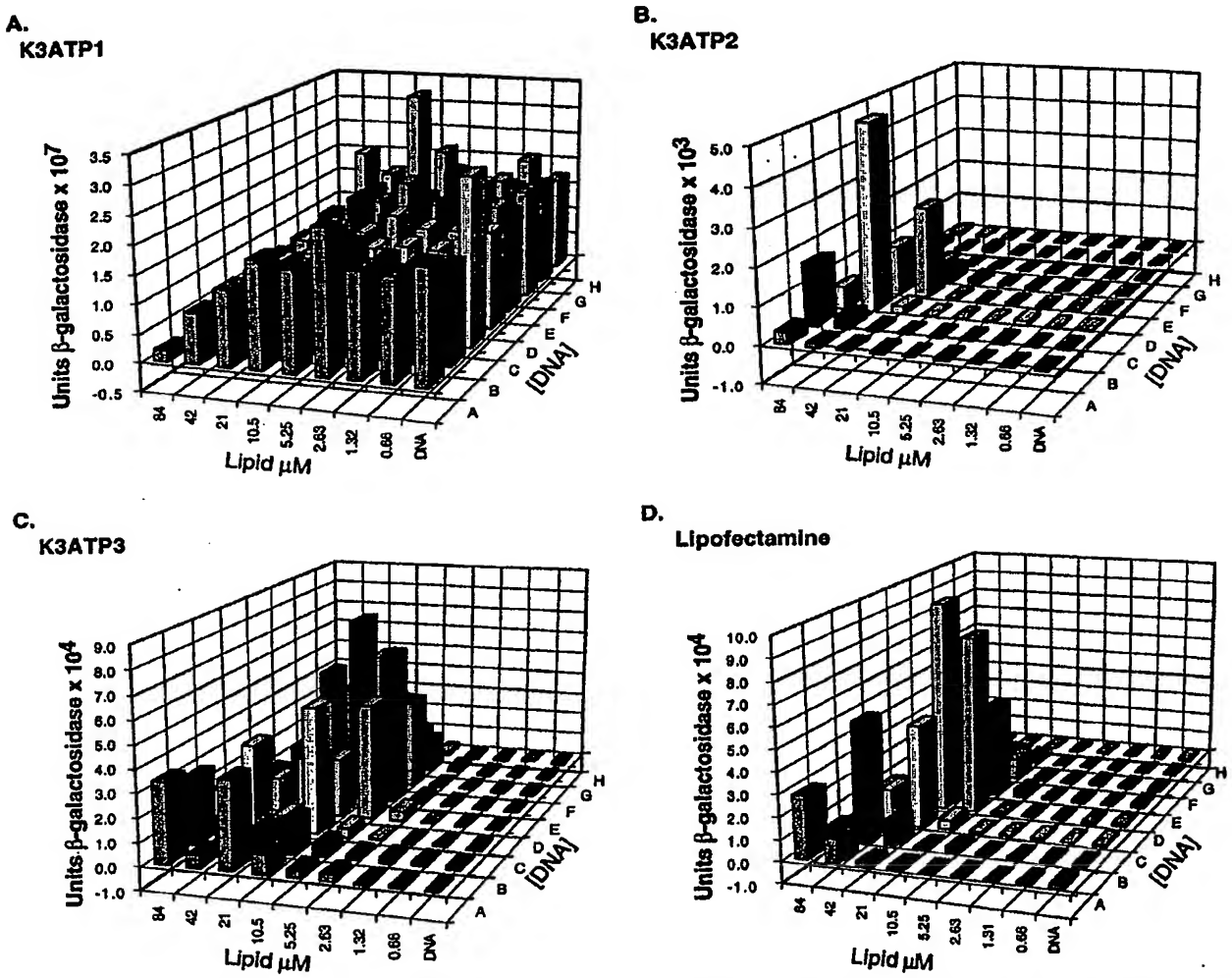


Fig. 3. Transfection of CHO cells. Three-dimensional histogram representations of 96-well transfection plates of (A) K_3ATP_1, (B) K_3ATP_2, (C) K_3ATP_3 and (D) Lipofectamine, showing the different range of tolerances of DNA and lipid combinations which will effectively transfect CHO cells. These graphs display not only the different regions where the peak transfection occurs but also the broadness of the range of effective transfection combinations using different reagents. Y axis displays units of β-galactosidase, X axis the range of cationic lipid concentrations in doubling dilutions from 84 μM to 0.66 μM. 'DNA' is the DNA-alone control. The Z axis is the range of DNA concentrations in doubling dilutions from A = 60.6 μM to H = 0.48 μM nucleotides.

main), 'Y' (spacer domain) and 'R1 to R3' (hydrophobic domain).

3.2. Demonstration of transfection

Transfection experiments were carried out using one or more of the plasmids pPGKlacZNLS, pCIGAL or pSVGAL carrying a gene cassette encoding the production of *E. coli* β-galactosidase. Forty-eight hours after transfection, the cytotoxicity of the transfection complexes was measured using the MTS assay, then transfection efficiency was measured by assaying lysates of the same cells for β-galactosidase as described above. Transfection was compared to that achieved with the commercial agents Lipofectamine or DMRIE-C (Life Technologies).

Fig. 3 shows the dramatic effect on transfection in CHO cells of changing the number of R groups in the hydrophobic domain of the molecule. Holding the rest of the molecule constant with three lysine (K_3) moieties, and an alanine spacer, the number of palmitoyl molecules was varied from one to three. The effect of this variation on transfection was that although the K_3ATP_{1-3} molecules all display some level of transfection of CHO cells above naked DNA, conjugates carrying two or three palmitoyl groups show peak transfection activities 4 and 3 orders of magnitude above this background control respectively.

These data further demonstrated that the dipalmitoyl molecule not only gave the highest levels of transfection of the three variants, but also gave a level of transfection significantly greater than that mediated by Lipofectamine. This was true whether measured as the 'peak' level of transfection, or the 'summed' data as shown in Table 1.

The differences between compounds can thus be observed by more than one parameter when testing in the 96-well plate assay. First, the maximal level of transfection can be observed in a single well of the 8×8 matrix. This designates the optimal lipid:DNA ratio and concentration for transfection mediated by the lipid in that cell type. Second, the summed data indicate how much variation from that optimal combination of lipid and DNA can be tolerated and still result in significant levels of transfection. This is illustrated graphically in the 3D histograms of Fig. 3 where in the case of the P_3 molecule peak transfection levels are significantly down relative to the P_2 molecule and similar to Lipofectamine. However, as shown in Table 1, the P_3 molecule shows a sum substantially greater than its peak (sum/peak = 7) whereas the P_2 molecule results show relatively close sum and peak values (sum/peak = 3.5), while Lipofectamine is intermediate (sum/peak = 5.4). The inference from this is that as the sum/peak value approaches 1, the range of useful transfection conditions becomes increasingly narrow. Conversely as the value increases, the useful range of conditions can be seen as broadening, with the codicil that when both transfection values are close to background, as in the P_1 results, the value of sum/peak can be misleading.

3.3. Cytotoxicity

General cytotoxicity over the entire range of lipid/DNA combinations was low. Cell viability measured using the MTS assay on the same dish of cells subsequently assayed for β-galactosidase gene product indicated that cell survival averaged across the plate was at least as high as when using Lipofectamine on the same cells (Fig. 4A). Particularly, the dipalmitoyl molecule showed a good level of viability of transfected cells compared to Lipofectamine.

For in vitro transfections, perhaps the more relevant data is the level of cytotoxicity at the specific

Table 1
Transfection of CHO cells

Reagent	Peak (units β-gal)	S.E.	Sum (units β-gal)	S.E.	Sum/peak
Lipofectamine	1.92×10^{-3}	0.048×10^{-3}	1.036×10^{-2}	0.07×10^{-2}	~5.4
K_3ATP_1	1.03×10^{-6}	0.566×10^{-6}	1.519×10^{-5}	0.27×10^{-5}	14.7
K_3ATP_2	4.76×10^{-3}	0.710×10^{-3}	1.667×10^{-2}	0.21×10^{-2}	3.5
K_3ATP_3	8.32×10^{-4}	0.417×10^{-4}	5.835×10^{-3}	2.18×10^{-3}	7.0

Results shown are the mean 'peak' or 'sum' data from four separate experiments. Absolute values obtained from each separate experiment were used to obtain mean data and standard error (S.E.) values.

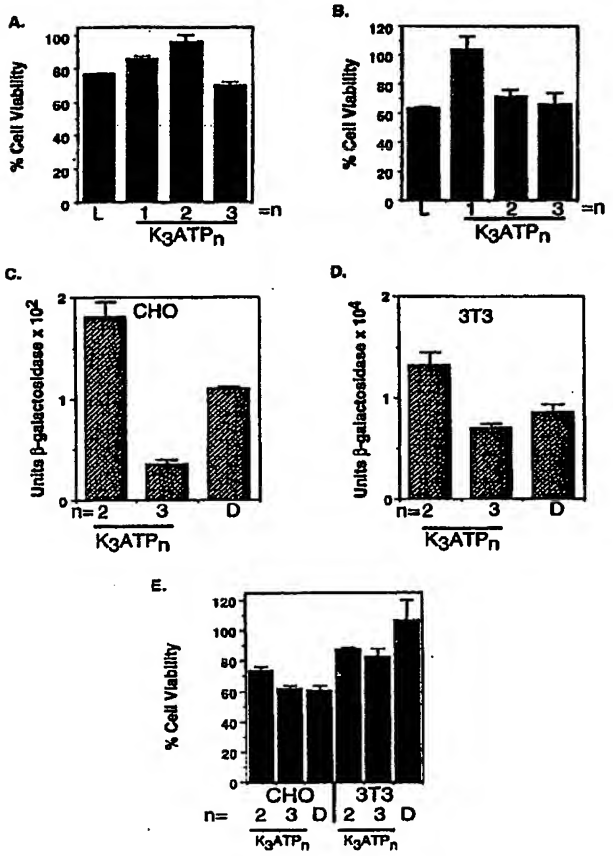


Fig. 4. Viability of transfected CHO cells. (A) Total plate i.e. 64 wells summed. (B) Viability of well displaying maximum transgene expression. In both A and B levels were referred to DNA-alone controls (Σ or mean of eight wells) as 100% viability. Viability data over four separate experiments were used. S.E. bars are shown. Peak transfection in CHO (C) and 3T3 cell lines (D). Relative transfection mediated by K_3ATP_2 (2), K_3ATP_3 (3), DMRIE-C (D) are shown. Optimal, or 'peak' transfection is displayed. S.E. bars are shown. (E) Cell viability under conditions of optimal transfection. 100% viability as in A and B, for each cell type. S.E. bars are shown. K_3ATP_2 (2), K_3ATP_3 (3), DMRIE-C (D).

combination of lipid and DNA which gives the most effective transfection. These are shown in Fig. 4B where it can be seen that at optimal transfection conditions, all the variants are still enabling greater than or equal to the cell viability observed when using Lipofectamine.

3.4. Other cells

Transfection activity of the K_3ATP_2 and K_3ATP_3 conjugates is not limited to CHO cells. Mouse 3T3 and CHO cells were grown over the same period and transfected under essentially identical conditions, with the exception that the transfection components (lipid and DNA) were diluted in serum free DME medium for 3T3 cells rather than the EMEM used for CHO cells.

Fig. 4C and D show that the two cell lines displayed a similar profile of optimal transfection with the different test lipids and DMRIE-C, although the actual level of transgene expression was reduced in the 3T3 cells compared to CHO cells. Viability of the cells remained good with all compounds in 3T3 cells, as shown in Fig. 4E. However, while it is interesting to observe that K_3ATP_2 was the most effective facilitator of transfection of both cell types, it remains possible that other cell lines may display a different reagent preference. It has been reported by others that different cell lines can display optimal transfection with different reagents [16].

3.5. Electron microscopy

Negative staining of lipid formulations demonstrated that the Tris conjugate assembly resembled branching stacks of discs which were relatively small in size (15–20 nm in width) as shown in Fig. 5A. The size estimate of a lipid bilayer made by Lasic [30] of 4 nm would indicate that these discs may in fact be constructed of a squashed sphere of lipid bilayer, described by Lasic as an oblate micelle. A comparison with the commercial DOSPA:DOPE formulation Lipofectamine in Fig. 5B shows a distinctively different structure of large multilamellar liposomes of larger dimensions (26–100 nm in diameter).

4. Discussion

Cationic lipids are well established as facilitators of nucleic acid uptake in mammalian cells. Advantages of using these types of molecules over viruses for delivery lie mainly in the low toxicity of the preparations (reviews [17,18]). This includes the reduced risks associated with immunogenicity, oncogenicity

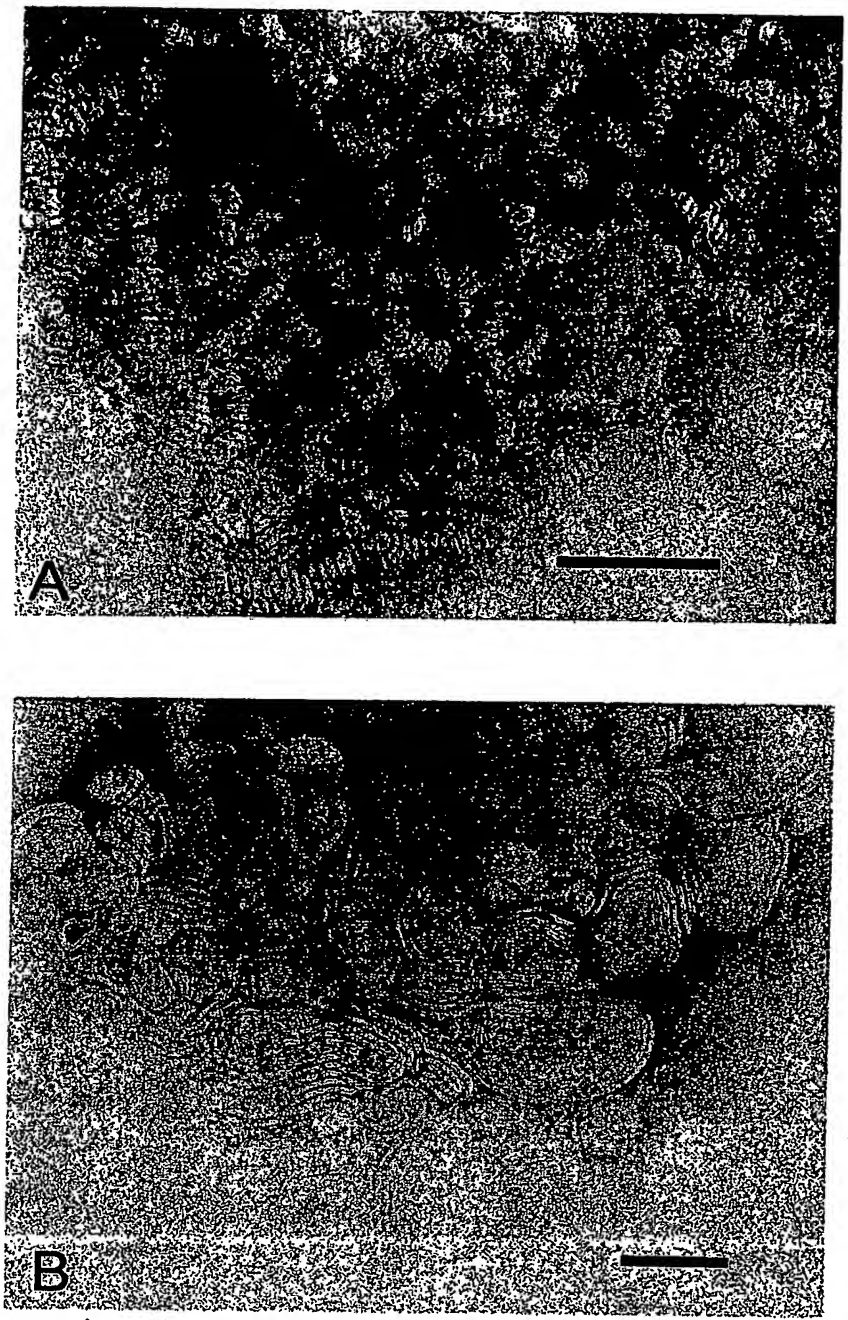


Fig. 5. Transmission electron micrographs of negatively stained (A) K_3ATP_3 and (B) Lipofectamine. Stock samples of liposomes at 2 mM were diluted 1 in 10 with filtered distilled water, and mixed 1:1 with filtered negative stain (ammonium molybdate 2%, pH 6.5) and spread onto carbon-formvar coated copper/rhodium 200 mesh electron microscope grids, and examined in a Jeol JEM 100CX electron microscope at 60 kV at original magnifications of (A) 33K and (B) 50K. Micrographs have been photographically enlarged to the same final magnification. Size bars: A = 90 nm; B = 60 nm.

and viraemia resulting from chance viral recombination events.

We have discovered a generic technology which has enabled us to make a class of cationic lipid transfection compounds whose chemical composition and mode of synthesis offer the ability to make alterations to the chemical properties of these compounds in a systematic fashion with relative ease. The work described here demonstrates one biological application of compounds utilising this unique Tris linkage of peptides to fatty acyl derivatives [9] in their use as transfection reagents.

The three symmetrical hydroxyl groups on the Tris molecule provide the opportunity to produce unique molecules without any stereoisomers. The nature of the fatty acyl chains at these positions can be readily varied and need not be identical. Most previously described synthetic cationic lipid molecules used for transfection incorporate two fatty acyl groups or a stereol backbone. This new cationic lipid family was designed so that the number and structure of the hydrophobic moieties could easily be changed from a single chain through to a maximum of three which has provided dramatic changes in their biological application.

The head group in the molecules reported here consists of a trilysine peptide producing four positive charges. Although not yet investigated, the number of charges in the head group could be varied by conjugating peptides carrying a different number of lysines to the hydrophobic backbone. The ability to readily change the number of charges in the head group provides the opportunity to systematically correlate the effect of charge in these molecules on their interaction with DNA and with the capacity to transfect genes into cells.

The three different peptide-Tris fatty acyl conjugates K_3ATP_1, P_2 and P_3 were synthesised in a single esterification reaction conjugating palmitic acid to the Boc(K-Cbz)$_3$ AT and then separated by HPLC and silica chromatography. However, each of these conjugates can also be prepared separately by generating the building blocks Boc(K-Cbz)$_3$OSU and ATP_1, ATP_2 or ATP_3 and subsequently coupling the polar head group to the hydrophobic group. The latter method is preferred when one of the conjugates is of particular interest and for scaling up production.

As a rule, with most transfection molecules described in the literature or commercially available, including Lipofectamine and DMRIE-C, transfection is reliant heavily on the combination of the active molecule with a neutral lipid, usually DOPE and occasionally cholesterol [14,19–26]. These lipids are used to support better liposome formation, and may enhance endocytic uptake [27,28]. Also, DOPE in particular has been shown to have a major role in destabilising the endosomal membrane by inducing a lamellar-hexagonal phase transition to aid in escape of DNA into the cytosol before lysosomal degradation occurs [29,30]. A few exceptions to this rule have been described. One by Wheeler et al., where the change of an alcohol to an amine in βAE-DMRIE obviated the need for a co-lipid [31] and another where the lipospermine DOGS (Transfectam) did not benefit from the addition of DOPE [32].

Unlike most cationic lipids, the transfection activity of the Tris conjugates was independent of formulation with any co-lipid, but rather relied on their own capacity to self-assemble into membranous structures in aqueous solution as demonstrated by K_3ATP_3 in Fig. 5A. This type of lipid assembly is notably different from that of Lipofectamine (DOSPA:DOPE 3:1) shown in Fig. 5B. As postulated with DOGS [32], the Tris molecules must at least partly incorporate the function of the fusogenic co-lipids themselves, as they do not benefit from their addition (data not shown). This non-requirement of the co-lipid removes the need for deliberate liposome formulation and greatly simplifies the use of these compounds.

The set of molecules used in this study has an ester bond between the hydrophobic fatty acyl backbone and the Tris linker which can be expected to have desirable biodegradability and biocompatibility after transfection into cells, and therefore less cytotoxicity. In fact, cytotoxicity indications were favourable compared to commercial reagents and levels of cell viability at optimal levels of transfection were particularly encouraging.

Although our data shown clearly show that the P_2 compound conferred higher transfection levels under optimal conditions compared to the P_3 compound in at least two cell types, the P_2 compound exhibited high efficiency in a narrow lipid:DNA ratio and concentration range, whereas the P_3 compound was

quite effective over a broad range. In the case of cell types more refractory to transfection, or in vivo application, this broad tolerance may be a great advantage.

The prototype Tris conjugate cationic lipids that we have described show great promise as gene delivery agents, with the P_2 compound already showing transfection activity that is highly competitive with two leading transfection reagents, Lipofectamine and DMRIE-C which are in common laboratory use. The different results obtained with the P_1, P_2 and P_3 variants provide a strong indication of the potential for changing biological characteristics by structural variation at the modular regions of the molecule.

Compared to viruses, cationic lipid reagents available currently are quite inefficient, resulting in relatively low levels of transfection. Despite such problems, sufficient groundwork has been undertaken to establish the in vivo potential of cationic lipids for gene transfer (reviewed by Scheule and Cheng [33]). Recent work [34] also argues that almost 100% of cells take up the delivered nucleic acid. The barrier to successful transfection is therefore probably not entry into the cell, which is facilitated efficiently by cationic lipids, but rather proper transport to the nucleus and processing of the information. This indicates an area for potentially increased efficiency for this type of transfection resulting from enhanced utilisation of genetic material taken in to the cell, whether this results from manipulation of the chemical structure of transfection molecules such as described here, or the addition of other material such as fusogenic (e.g. [35]) or targeting peptides (review [36]). The peptide nature of the hydrophilic domain of the Tris conjugate molecules also provides an opportunity to incorporate such peptides with functional domains into the compounds for a further level of versatility.

The Tris-linked reagents thus lend themselves to systematic modification of the three modular regions, namely cationic head group ('x'), spacer ('y') and hydrophobic domains (R1 to R3) (in Fig. 1), and evaluation of the impact of those changes on transfection activity would be expected to give a broad range of transfection opportunities from which top performers for specific applications could be chosen.

Recent efforts in our laboratory are currently focussed on synthesising and testing further variants at the specified variable domains.

Acknowledgements

The excellent technical help of Ms Denise Lewy in generating the plasmid pCIGAL is acknowledged by the authors for help.

References

[1] F. Ledley, Curr. Opin. Biotechnol. 5 (1994) 626–636.
[2] B. Abdallah, L. Sachs, B.A. Demeneix, Biol. Cell 85 (1995) 1–7.
[3] X. Gao, L. Huang, Biochem. Biophys. Res. Commun. 179 (1991) 280–285.
[4] E.R. Lee, J. Marshall, C.S. Siegel, J. Canwen, N.S. Yew, M.R. Nichols, J.B. Nietupski, R.J. Ziegler, M.B. Lane, K.X. Wang, N.C. Wan, Scheule, D.J. Harris, A.E. Smith, S.H. Cheng, Hum. Gene Ther. 7 (1996) 1701–1717.
[5] S. Ferrari, E. Moro, A. Pettenazzo, J.P. Behr, F. Zacchello, M. Scarpa, Gene Ther. 4 (1997) 1100–1106.
[6] K. Kaneko, A. Tsukamoto, Cancer Lett. 107 (1996) 211–215.
[7] J. Zabner, Adv. Drug Deliv. Rev. 27 (1997) 17–28.
[8] J. Smith, Y. Zhang, R. Niven, J. Adv. Drug Deliv. Rev. 26 (1996) 135–150.
[9] R.G. Whittaker, P.J. Hayes, V.J. Bender, Peptide Res. 6 (1993) 125–128.
[10] G.N. Hannan, S.A. Lehnert, E.S. MacAvoy, P.A. Jennings, P.L. Molloy, Gene 130 (1993) 233–239.
[11] M.J. Sleigh, T.J. Lockett, EMBO J. 4 (1985) 3831–3837.
[12] S.K. Shapira, J. Chou, F.V. Richaud, M.J. Casadaban, Gene 25 (1983) 71–82.
[13] C.V. Hall, P.E. Jacob, G.M. Ringold, F. Lee, J. Mol. Appl. Genet. 2 (1983) 101–109.
[14] J.H. Felgner, R. Kumar, C.N. Sridhar, C.J. Wheeler, Y.J. Tsai, R. Border, P. Ramsey, M. Martin, P.L. Felgner, J. Biol. Chem. 269 (1994) 2550–2561.
[15] M. and A. Bodanszky, Principles of Peptide Synthesis, Springer-Verlag, Berlin, 1984.
[16] D. Wang, N.H. Jing, Q.S. Lin, Biochem. Biophys. Res. Commun. 226 (1996) 450–455.
[17] D.D. Lasic, N.S. Templeton, Adv. Drug Deliv. Rev. 20 (1996) 221–266.
[18] R.J. Lee, L. Huang, Crit. Rev. Ther. Drug Carr. Syst. 14 (1997) 173–206.
[19] I. VanDerWoude, A. Wagenaar, A.A.P. Meekel, M.B.A. TerBeest, M.H.J. Ruiters, J.B.F.N. Engberts, D. Hoekstra, Proc. Natl. Acad. Sci. USA 94 (1997) 1160–1165.
[20] M.J. Lee ER, C.S. Siegel, C. Jiang, N.S. Yew, M.R. Nichols, J.B. Nietupski, R.J. Ziegler, M.B. Lane, K.X. Wang, N.C.

Wan, R.K. Scheule, D.J. Harris, A.E. Smith, S.H. Cheng, Hum. Gene Ther. 7 (1996) 1701–1717.
[21] L.K. Lewis JG, A. Kothavale, W.M. Flanagan, M.D. Matteucci, R.B. DePrince, R.A. Mook Jr., R.W. Hendren, R.W. Wagner, Proc. Natl. Acad. Sci. USA 93 (1996) 3176–3181.
[22] D.J. Stephan, Z.Y. Yang, H. San, R.D. Simari, C.J. Wheeler, P.L. Felgner, D. Gordon, G.J. Nabel, E.G. Nabel, Hum. Gene Ther. 7 (1996) 1803–1812.
[23] T. Paukku, S. Lauraeus, I. Huhtaniemi, P.K. Kinnunen, Chem. Phys. Lipids 87 (1997) 23–29.
[24] W.S. Zhao DD, J.T. Lee, S. Kouchi, H. Ohmori, T. Yasuda, Acta Med. Okayama 51 (1997) 149–154.
[25] S. Obika, W. Yu, A. Shimoyama, T. Uneda, K. Miyashita, T. Doi, T. Imanishi, Bioorg. Med. Chem. Lett. 7 (1997) 1817–1820.
[26] J.Y. Legendre, A. Trzeciak, D. Bur, U. Deuschle, A. Supersaxo, Pharm. Res. 14 (1997) 619–624.
[27] M.J. Bennett, M.H. Nantz, R.P. Balasubramaniam, D.C. Gruenert, R.W. Malone, Biosci. Rep. 15 (1995) 47–53.
[28] S.W. Hui, M. Langner, Y.-L. Zhao, P. Ross, E. Hurley, K. Chan, Biophys. J. 71 (1996) 590–599.
[29] H. Farhood, N. Serbina, L. Huang, Biochim. Biophys. Acta Biomembr. 1235 (1995) 289–295.
[30] D.D. Lasic, Liposomes in Gene Delivery, CRC Press, New York, 1997, pp. 145–150.
[31] C.J. Wheeler, L. Sukhu, G.L. Yang, Y.L. Tsai, C. Bustamente, P. Felgner, J. Norman, M. Manthorpe, Biochim. Biophys. Acta Biomembr. 1280 (1996) 1–11.
[32] K.A. Remy, J.S., V. Mordvinov, F. Schuber, J.P. Behr, Proc. Natl. Acad. Sci. USA 92 (1995) 1744–1748.
[33] R.K. Scheule, S.H. Cheng, in: P.L. Felgner, M.J. Heller, P. Lehn, J.P. Behr, F.C. Szoka Jr. (Eds.), Artificial Self Assembling Systems for Gene Delivery Conf. Proc. CHI, Am. Chem. Soc., Washington, DC, 1996, Ch. 17, pp. 177–190.
[34] Z. Bebok, A.M. Abai, J.Y. Dong, S.A. King, K.L. Kirk, G. Berta, B.W. Hughes, A.S. Kraft, S.W. Burgess, W. Shaw, P.L. Felgner, E.J. Sorscher, J. Pharmacol. Exp. Ther. 279 (1996) 1462–1469.
[35] H. Kamata, H. Yagisawa, S. Takahashi, H. Hirata, Nucleic Acids Res. 22 (1994) 536–537.
[36] E. Forssen, M. Willis, Adv. Drug Deliv. Rev. 29 (1998) 249–271.